

Strain specific immunostimulatory potential of lactobacilli-derived exopolysaccharides

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Abstract

*Lactobacilli, the most commonly used probiotics, show strain specific immunostimulatory effects. The strain specificity is related to the structure of cell-wall components such as lipoteichoic acids (LTAs), ligands for TLR2. In contrast, the role of exopolysaccharides (EPSs), the major components of lactobacilli biofilm, in the "cross-talk" between bacteria and the immune system is poorly documented. In this study, we have tested immunomodulating potential of lactobacilli derived EPSs, by their ability to modulate in vitro production of cytokines by murine peritoneal macrophages. We have examined EPSs of three lactobacilli strains (*L. reuteri*, *L. johnsonii*, *L. animalis/murinus*) isolated from colitic mice. The effect was compared with that of whole bacteria and LTA. All three nonviable *Lactobacillus* strains induced high amounts of cytokines with a strain specific profile. In contrast, the tested exopolysaccharides, exert low, albeit highly strain diversified stimulatory activities. The immunostimulatory potential of EPSs was correlated with the interaction strength between EPS and macrophage receptors as measured by atomic force spectroscopy.*

Key words: *lactobacilli, probiotics, exopolysaccharide (EPS), lipoteichoic acid (LTA), macrophages, cytokines.*

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Introduction

Over the past few years there is increasing interest in using probiotic bacteria, mostly *Lactobacillus* species (whole microorganisms or cell wall components) in a treatment and prevention of diseases with altered immune responses (allergic, autoimmune and chronic inflammatory diseases). The therapeutic effect is strain specific and depends on immunoregulatory properties of the probiotic used [1-3]. It has been shown that some *Lactobacillus* strains can directly activate host immune cells. However, the bacterial cell components and corresponding receptors on the immune cells responsible for the induction of cytokine production have not yet been fully determined. On the other hand, it is accepted that innate immune responses are induced upon recognition of pathogens by

pattern recognition receptors, which include the Toll-like receptors (TLRs) and nucleotide binding oligomerization domain proteins, NOD1 and NOD2 [4, 5]. A substantial amount of evidence has accumulated that lipoteichoic acids (LTAs), ligands for TLR2 and peptidoglycans, ligands for NOD2, play a crucial role in the induction of immune response by probiotic *Lactobacillus* strains [5, 6]. On the contrary, there is a scarce knowledge concerning the immunostimulatory properties of exopolysaccharides (EPSs) produced by lactobacilli. On the other hand, there has been increasing interest in bacterial EPSs due to their biological activities and advantages over the polysaccharides that are currently in use [7-9]. It has been shown that EPS, a major component of bacteria biofilm, protects bacterial cells from desiccation, phage attack, antimicrobial

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compounds, osmolytic stress and predatory attack from protozoa [10-12].

The present study has been focused on analyzing the immunoregulatory capacity of crude EPSs produced by lactic bacteria (*L. reuteri*, *L. johnsonii*, *L. murinus*) by determination of the profile of inflammatory cytokines released by the *in vitro* activated macrophages. The immunostimulatory potential of the EPSs was compared with that of whole nonviable bacteria and with LTAs, isolated from the tested *Lactobacillus* strains.

Materials and methods

Bacteria

We have selected three *Lactobacillus* strains – *L. reuteri* 115 (L.115), *L. johnsonii* 142 (L.142), *L. animalis/murinus* 148 (L.148) for the study. They were chosen from a collection of bacterial strains previously isolated from the gastrointestinal tracts of α 2-deficient mice which showed clinical symptoms of colitis [13]. They were constituents of the Modified Schaedler Flora colonizing the gut of this mice model, kept in SPF conditions. Phenotypic and genotypic identification of lactobacilli was performed using commercial identification systems (API50CHL, bioMérieux, France) and DNA sequencing of variable regions of the 16S rRNA [14]. The selected micro-organisms were stored lyophilized until used. The strains were then prepared according to Christensen procedure [1]. Briefly, each *Lactobacillus* strain was initially cultured in 10 ml of MRS broth (Oxoid, UK) for 16 h at 37°C in anaerobic conditions and then transferred into 500 ml of fresh MRS broth and incubated for 8 h in the same conditions. The bacterial growth was sedimented by centrifugation at 2000 rpm for 15 min, washed three times with phosphate buffer solution (PBS, pH 7.2) and finally, suspended in 50 ml of PBS and frozen at –80°C. Before freezing, numbers of bacteria in the samples were estimated by the standard viable count method in cfu/ml. The frozen bacteria were killed by gamma irradiation using a dose of 10 kGy (1 Mrad). After killing, samples of the bacterial suspension were checked for viability by culture in MRS broth followed by plating on MRS agar (Oxoid, UK). Finally they were freeze-dried and weighed. One mg of the killed lyophilized suspension contained approximately 10⁹ bacterial cells. Before use, the bacteria were rehydrated in PBS buffer.

Preparation of exopolysaccharides

Exopolysaccharides were extracted from the *Lactobacillus* strains as described before [15]. Briefly, viable bacterial cells were stored at –70°C in MRS broth supplemented with 10% glycerol. Bacteria were cultivated in supplemented MRS liquid broth (Oxoid, UK) under anaerobic conditions at 37°C for 48 h. Cells were harvested by centrifugation at 8000 rpm (4°C, 30 min) and washed twice

with PBS. Bacterial mass was suspended in water (10 ml) and sonicated three times for 5 min, in an ice bath. After the centrifugation at 6000 rpm (30 min, 4°C), the supernatant was centrifuged twice at 12 000 rpm at 4°C for 1 h and then precipitated with 5 volumes of cold ethanol (–20°C, overnight). The precipitated material was recovered by centrifugation at 12 000 rpm, 4°C for 20 min and freeze-dried. Alternatively, cell mass suspended in water, homogenized by ultrasonication for 5 min was extracted with trichloroacetic acid (TCA, 10% of final concentration) with magnetic stirring at room temperature for 2 h. The suspension was centrifuged and the supernatant after precipitation with 5 vol. of ethanol (–20°C, 18 h) was centrifuged (8000 rpm, 4°C, 30 min), solubilized in water, dialyzed and lyophilized. Purification of EPS was performed by gel filtration on a column of TSK HW-55S (1.6 cm × 100 cm) in 0.1 M ammonium acetate or TSK HW-50 (1.6 cm × 100 cm) in 0.05 M aqueous pyridine acetate buffer (pH 5.6). The column eluate was monitored with a Knauer differential refractometer. The first fraction, which eluted in the void volume, contained polysaccharide and was the subject of the present investigation as crude EPSs.

Preparation of lipoteichoic acid

A defrosted aliquot of bacteria was mixed with an equal volume of n-butanol (Merck, Germany) under stirring for 30 min at room temperature. After centrifugation at 13 000 g for 20 min, the aquatic phase was collected and dialyzed against distilled water, lyophilized, resuspended with chromatography start buffer (15% n-propanol in 0.1 M ammonium acetate, pH 4.7, Merck, Germany) and centrifuged at 45 000 g for 15 min. The supernatant was subjected to hydrophobic interaction chromatography on octyl-Sepharose CL-4B (1.6 × 40 cm, Pharmacia Fine Chemicals, Sweden). Unbound material was washed out with 15% n-propanol in 0.1 M ammonium acetate buffer (pH 4.7). Afterwards bound materials were eluted with 35% n-propanol in 0.1 M ammonium acetate buffer (pH 4.7, Merck, Germany). Column fractions containing LTA were pooled and dialyzed against distilled water.

Mice

Inbred CBA/J female mice (8-12 weeks of age, 18-22 g) were maintained in the Animal Breeding Unit, Department of Immunology, Jagiellonian University Medical College, Krakow. All mice were housed 4-5 per cage in the laboratory room with water and standard diet *ad libitum*. The authors were granted permission by the Local Ethics Committee to use mice in this study.

Cells

Peritoneal mouse macrophages (M ϕ) were induced by intraperitoneal injection of 1.0 ml of paraffin oil (Sigma, USA). Cells were collected 72 h later by washing out the peritoneal cavity with 5 ml of PBS containing

5 U heparin/ml (Polfa, Poland). Cells were centrifuged and red blood cells were lysed by osmotic shock using distilled water; osmolarity was restored by addition of $2 \times$ concentrated PBS. The presence of macrophages (85-90%) was judged by cytochemical demonstration of non-specific esterase-positive mononuclear cells, using α -naphthyl acetate (Sigma, USA).

Cell cultures and treatment

M ϕ were cultured in 24-well flat-bottom cell culture plates at 5×10^5 /well in RPMI 1640 medium (JR Scientific Inc., USA) supplemented with 5% FCS, at 37°C in an atmosphere of 5% CO₂. Cells were activated either with nonviable bacterial cells used at a ratio 200 : 1 of bacteria : macrophages or with bacterial components (EPSs, LTA) used at concentrations indicated in the Legends to figures. After 24 h culture supernatants were collected and frozen at -80°C until used. Viability of the cells was routinely monitored by cellular exclusion of trypan blue.

Cytokines determination

Cytokine concentrations in culture supernatants were measured using sandwich ELISA as described previously [16]. For interleukin 6 (IL-6), IL-10, IL-12p40 microtiter plates (Corning, USA) were coated overnight with rat mAb against a mouse cytokine (capture antibody). After blocking the plates with 4% albumin (2 h), standards and tested supernatants were added and incubated overnight. Finally, biotinylated antibodies against the same cytokine were added for 1 h. The ELISA was developed using horseradish peroxidase conjugated with streptavidin (Vector, USA) followed with *o*-phenylenediamine and H₂O₂ (both Sigma-Aldrich, Germany). The reaction was stopped with 3 M H₂SO₄. The optical density of each sample was measured at 492 nm in a microplate reader. 0.05% Tween 20 in PBS was used as a washing solution. Recombinant murine cytokines were used as standard. For tumor necrosis factor α (TNF- α) 3% non-fat dried milk was used for blocking the plates. Hamster anti-mouse/rat TNF- α and biotinylated rabbit anti-mouse/rat TNF- α mAbs were used to determine presence of the cytokine.

The following reagents were used for the following assays:

- IL-6: Rat anti-mouse IL-6 and biotinylated rat anti-mouse IL-6 (both Pharmingen, USA) mAbs were used as capture and detecting antibodies. Recombinant mouse IL-6 (PeproTech, USA) was used as a standard.
- IL-10: Rat anti-mouse IL-10 and biotinylated rat anti-mouse IL-10 mAbs were used as capture and detecting antibodies. Recombinant mouse IL-10 was used as a standard (all reagents from Pharmingen, USA).
- IL-12p40: Rat anti-mouse IL-12 (p40rp70), clone 15.6 (Pharmingen, USA) mAb and biotinylated rat anti-mouse IL-12(p40), clone 17.8 (Endogen, USA) mAb were used

as capture and detecting antibodies. Recombinant mouse IL-12 (Genzyme, UK) was used as a standard.

- TNF- α : Hamster anti-mouse/rat TNF- α and biotinylated rabbit anti-mouse/rat TNF- α (both Pharmingen, USA) mAbs were used as capture and detecting antibodies. Recombinant mouse TNF- α (Sigma-Aldrich, Germany) was used as a standard.

Atomic force spectroscopy measurements

The rupture forces between a tip coated with bacterial components (A) and macrophages (B) seeded onto coverslips were measured using a commercial atomic force spectroscopy measurements (AFM) instrument, the Thermomicroscopes CP equipped with a multimode head and a 100 μ m scanner. The deflection of the cantilever was detected by a position sensing photodiode detector. All experiments were performed in PBS solution (pH 7.4) by using a commercial liquid cell (Thermomicroscopes).

(A) Tip functionalized with bacterial components (EPSs)

Standard V-shaped silicon nitride (Si₃N₄) cantilevers (Microlevers, Veeco) with a nominal spring constant of 0.01 N/m were used. The tips were functionalized with EPSs as described previously [17].

(B) Macrophage cell preparation

The cells $1-2 \times 10^6$ /ml were suspended in PBS solution containing 100 ml of 2% BSA. Next, they were put on a glass coverslip and incubated at 37°C in 5% CO₂ for 2 h. Afterwards, the glass coverslip with macrophages was rinsed in PBS and immersed in 1.5% glutaraldehyde PBS solution for 1 min. Glutaraldehyde causes stabilization of receptors in the cell membrane. After 1 min the coverslip was rinsed in PBS and immediately used for measurements.

Statistic analysis

Student's *t*-test was used to determine the significance of group differences. Results are expressed as mean \pm SEM values. A *P*-value less than 0.05 was considered statistically significant. Analysis were performed using Graphpad Prism v. 5.01 (GraphPad Software, USA).

Results

Effect of bacterial exopolysaccharides on the release of cytokines by macrophages

Mouse peritoneal macrophages elicited *in vivo* with paraffin oil, not stimulated *in vitro* produce small amounts of pro-inflammatory cytokines (TNF- α , IL-6, IL-12p40) and undetectable amounts of IL-10. However, incubation of these macrophages with nonviable lactobacilli, *L. reuterii* 115, *L. johnsonii* 142 and *L. animalis/murinus* 148, resulted in a massive strain specific release of a variety of pro- and anti-inflammatory cytokines.

To determine whether EPSs from these lactobacilli strains show immunoregulatory activities, macrophages have been incubated with EPSs and the profile of cytokines has been determined in culture supernatants. Immunoregulatory potential of EPS varies among the lactobacilli strains, as shown in Figs. 1, 2, 3. All three EPSs preparations were much less effective than whole nonviable bacteria. Moreover, EPSs from distinct lactobacilli strains induced different pattern of cytokines, as indicated by different ratio of pro- to anti-inflammatory cytokines (Table 1). Exopolysaccharide 115 was the best stimulator of pro-inflammatory cytokines (IL-6 and TNF- α) among the tested EPSs. The comparison of cytokine production after EPSs stimulation with the effect of nonviable bacterial cells of their origin shows that EPS 115 contributes significantly to the production of TNF- α (34% production of TNF- α stimulated by *L. reuteri* 115) and IL-6 (84%). On the other hand, EPS 142 shows the highest impact on the induction of IL-12p40 (45% production of IL-12 released after stimulation with nonviable *L. johnsonii* 142). Interestingly, all

tested EPSs stimulated negligible amounts of IL-10, as compared to the effect of whole bacteria cells.

Atomic force spectroscopy measurements of the interaction strength between exopolysaccharide and macrophage receptors

In order to determine an average force required to break the bonds which are characteristic for a particular bacterial antigen-macrophage complex, a set of force-distance curves was collected using the AFM tip coated with the particular kind of antigen. Those curves represent dependences of the cantilever deflection on distance between the end of the tip and the macrophage membrane [17]. The rupture of the antigen-receptor bond corresponds to a characteristic peak on the curve. The heights of those adhesive peaks represent the force required to destroy the antigen-receptor bond. The average rupture force value is determined from the force histograms extracted from a family of force-distance curves (400) reflecting the statistical character of the bond breaking

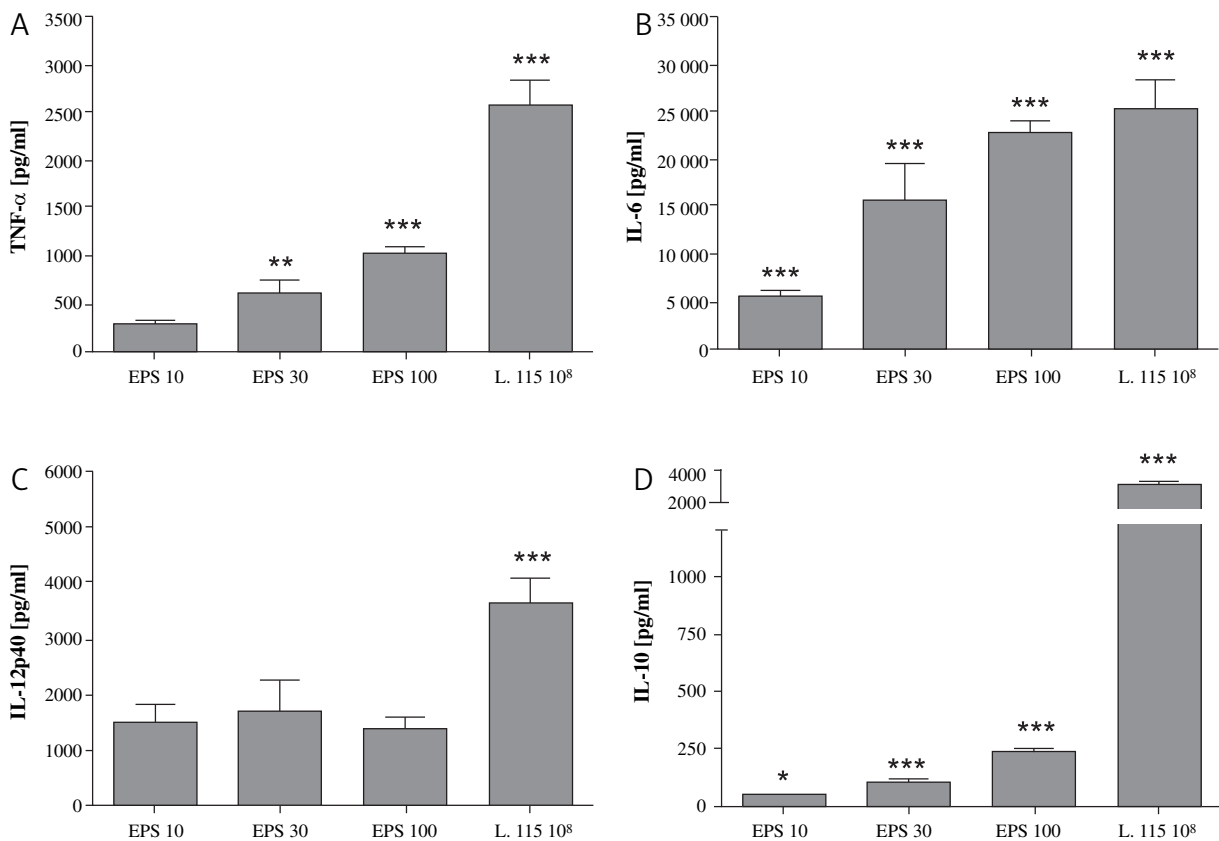


Fig. 1. Cytokine secretion from macrophages induced by EPS 115 and *L. reuteri* 115. Peritoneal macrophages (5×10^5 /well) were incubated with different concentrations of EPS 115 (10-100 μ g/ml) or with nonviable bacterial cells at concentration of 10^8 cfu/well. The levels of TNF- α (A), IL-6 (B), IL-12p40 (C) and IL-10 (D) were measured by ELISA in the culture supernatants after 24 h of incubation. Data are mean \pm SEM values derived from at least three independent experiments, each based on macrophages isolated from four mice and tested in duplicate; * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, treated versus untreated macrophages

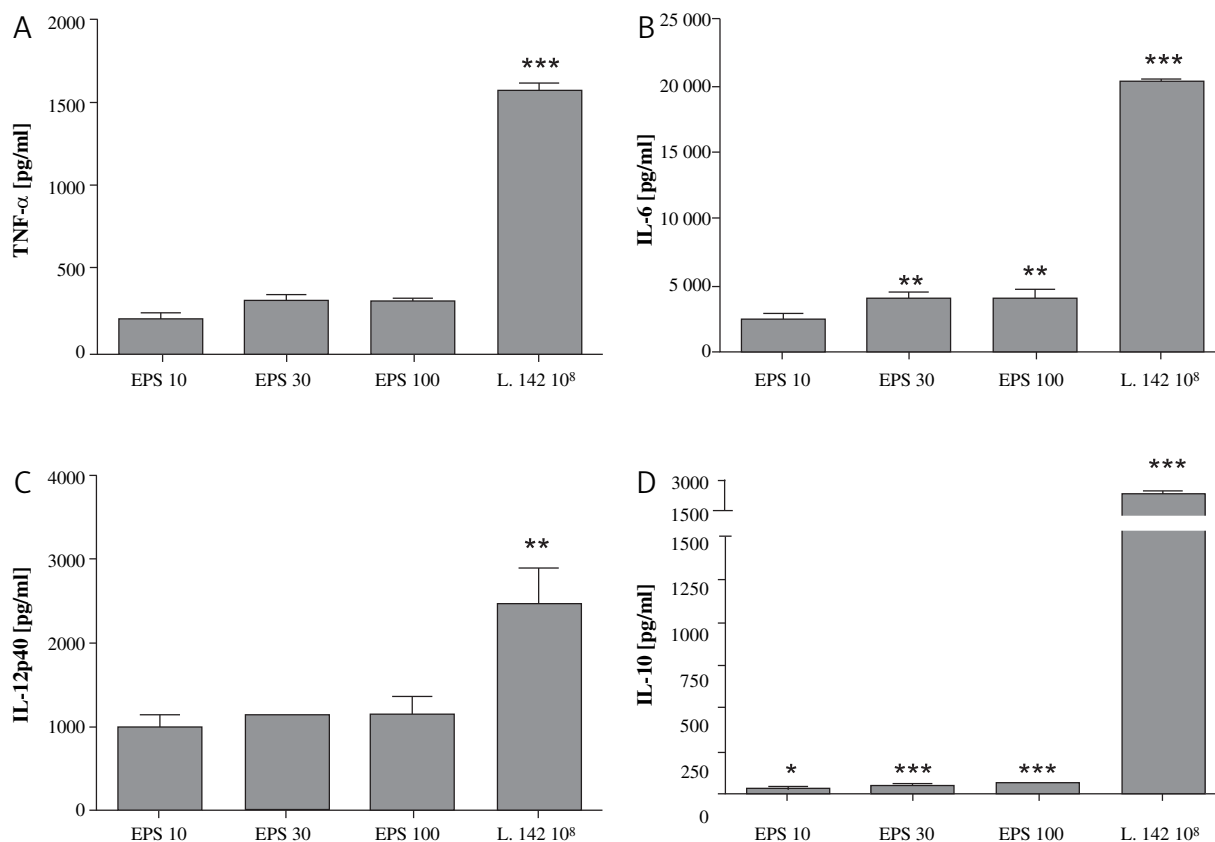


Fig. 2. Cytokine secretion from macrophages induced by EPS 142 and *L. johnsonii* 142. Peritoneal macrophages (5×10^5 /well) were incubated with different concentrations of EPS 142 (10-100 $\mu\text{g/ml}$) or with nonviable bacterial cells at concentration of 10^8 cfu/well. The levels of TNF- α (A), IL-6 (B), IL-12p40 (C) and IL-10 (D) were measured by ELISA in the culture supernatants after 24 h of incubation. Data are mean \pm SEM values derived from at least three independent experiments, each based on macrophages isolated from four mice and tested in duplicate; * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, treated versus untreated macrophages

process. The histograms of rupture forces measured in this work are shown in Fig. 4. The histogram obtained for EPS 115-macrophage complex is shown in Fig 4A. Similarly to the previously described reference complex (LPS), this histogram also contains two peaks [17]. The first one can be attributed to a weak specific interaction, whereas the second one to a stronger specific interaction. The value of average rupture force for EPS 115-macrophage system is high and comparable with the value of rupture forces for the reference complex. However, less events of bond breaking were observed for EPS 115. It means that the number of active receptors for EPS 115 binding is smaller than for reference complex and, therefore, the specific interactions between EPS 115 and macrophage receptors are not frequent. On the other hand, the appearance of two peaks and the high value of mean rupture force indicate relatively high strength of such interaction. In Figs. 4B and 4C the histograms for EPS 142 and EPS 148-macrophage complexes are shown. In both cases only single peaks are visible. The average rupture force and the frequency of events for these systems are smaller

than for the previous one. Such results suggest that for EPS 142 and EPS 148 the affinity to macrophage receptors is much lower than those for the reference system and for EPS 115. Furthermore, positions of the first peaks on all histograms presented in Fig. 4 overlap. Based on previous results [17] we can conclude that this peak occurs in the small range of forces attributed to specific interactions between bacterial antigens and receptors with small affinity.

The effectiveness of exopolysaccharide to induce inflammatory mediators compared with lipoteichoic acid and whole nonviable bacteria

To answer the question whether EPS and LTA induce the same cytokine profile as bacteria of their origin, macrophages were stimulated with EPS and LTA at the concentrations of 100 $\mu\text{g/ml}$ or with bacteria (10^8 cfu/ml). *L. johnsonii* 142 was selected for these investigations, as detailed structure of EPS derived from this strain has been published recently [18]. Analysis of cytokine production by macrophages stimulated with these components showed

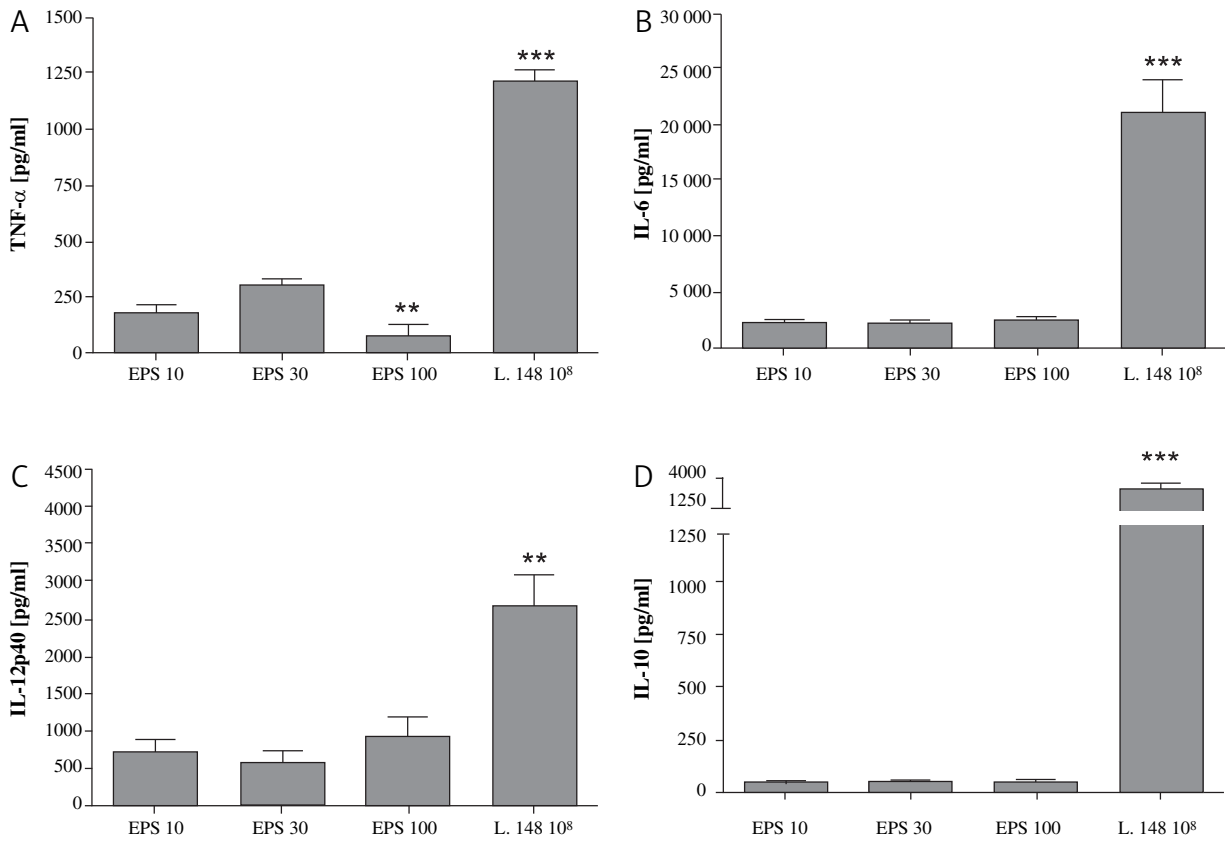


Fig. 3. Cytokine secretion from macrophages induced by EPS 148 and *L. animalis* 148. Peritoneal macrophages (5×10^5 /well) were incubated with different concentrations of EPS 148 (10-100 μ g/ml) or with non-viable bacterial cells at concentration of 10^8 cfu/well. The concentrations of TNF- α (A), IL-6 (B), IL-12p40 (C) and IL-10 (D) were measured by ELISA in the culture supernatants after 24 h of incubation. Data are mean \pm SEM values derived from at least three independent experiments, each based on macrophages isolated from four mice and tested in duplicate; ** $p < 0.005$, *** $p < 0.001$, treated versus untreated macrophages

Table 1. TNF- α /IL-10 and IL-12p40/IL-10 ratio in supernatants from macrophages induced by nonviable lactobacilli and related exopolysaccharides

Stimulation	Ratio	
	TNF- α : IL-10*	IL-12p40 : IL-10*
<i>L. reuteri</i> 115	1 : 1	1 : 1
EPS 115	4 : 1	6 : 1
<i>L. johnsonii</i> 142	1 : 1	1 : 1
EPS 142	3 : 1	14 : 1
<i>L. animalis/murinus</i> 148	1 : 1	1 : 1
EPS 148	1 : 1	11 : 1

*Ratio was calculated from the results shown in Figs. 1, 2 and 3.

that EPS and LTA induced the similar pattern of cytokines. However, the production of cytokine after LTA stimulation was higher than that of EPS. Importantly, both structures

were much less effective in cytokine induction when compared to whole bacteria (Fig. 5).

Discussion

Extracellular polymeric substances produced by microorganisms are a complex mixture of biopolymers primarily consisting of EPSs, proteins, nucleic acids and lipids [19]. The key function of EPS, a major component of bacterial biofilm, is protecting bacteria against environmental stress including immune attack. Lactobacilli, as well as other bacteria, produce different kinds of EPSs exhibiting a wide diversity of structures. It suggests that EPS show strain specific biological properties including diversified immunostimulatory potential.

To verify this hypothesis, in the present study we examined the immunostimulatory activities of EPSs from three different *Lactobacillus* strains (*L. reuteri* 115, *L. johnsonii* 142 and *L. animalis/murinus* 148), previously isolated from colitic mice [13].

In our study we demonstrated that macrophages activated *in vitro* by these nonviable bacteria released substantial amounts of both pro- and anti-inflammatory cytokines. However, some differences between lactobacilli strains in their capacity to induce synthesis of proinflammatory cytokines have been observed. The results confirmed our previous report which showed similar profile of cytokines induced by the tested *Lactobacillus* strains [20]. However, when macrophages were stimulated with EPSs, instead of whole bacterial cells, different patterns with significantly smaller amounts of all tested cytokines were observed. Importantly, there were clear differences among strains. For example, the most active EPS from *L. reuterii* 115 induced approximately 10 times more IL-6 than the least active EPS from *L. johnsonii* 148. Therefore, our data, along with other reports, suggest that lactobacilli may produce exopolysaccharides with either strong or negligible immunostimulatory potential. Great structural diversity of bacterial EPSs seems to be responsible for such unpredictable biological activities. Interestingly, the immunostimulatory potential of EPSs was correlated with the strength of interactions between macrophages and the bacterial cell-wall components, as shown by AFM. This observation may suggest the presence of still unknown EPS-specific receptor(s). Whether EPS is recognized by PRRs (pathogen recognition receptors) and how composition of structural carbohydrates correlates with their biological functions needs to be explained. Further studies are necessary to determine whether highly purified EPSs will show similar immunostimulatory properties with those exhibit by crude extracellular polymeric substances. Finally, we have asked the question whether EPS and LTA induce the same cytokine profile as bacteria of their origin? For this study we have selected *L. johnsonii* 142 strain, producer of EPS with known sugar composition [18]. Analysis of cytokine production by macrophages stimulated with these components showed that *L. johnsonii* 142, potentially beneficial intestinal bacteria [21], induced anti-inflammatory profile of cytokines, with the highest potential to induce IL-10. In contrast, EPS and LTA, despite distinct immunostimulatory potential, both induced pro-inflammatory profile of cytokines. We have described recently the similar effect of other EPS. Exopolysaccharide from *L. rhamnosus* KL37 effectively induced the production of macrophage cytokines, especially TNF- α IL-6 and IL-12. Its stimulatory potential was significantly lower than that of intact bacteria cells. However, effective concentrations of EPS were similar to immunostimulatory concentrations of LTA, the ligand for TLR2 [21, 22]. It suggests, that this EPS may contribute *in vivo* to the interaction between biofilm producing lactobacilli and the immune cells [19]. However, our findings also indicate that other EPSs induce production of low levels of cytokines of different profile than in case of whole bacterial cells. Thus, the contribution of

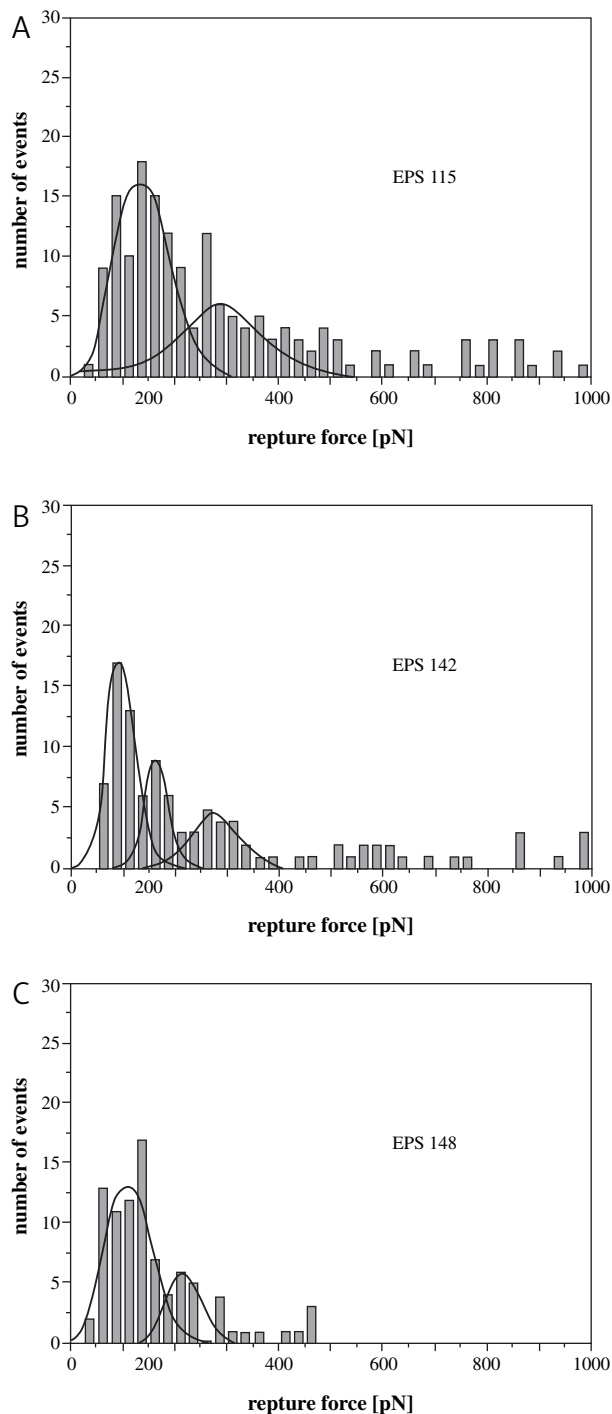


Fig. 4. AFS/AFM: Histogram of rupture forces measured between bacterial cell-wall structures (EPSs) and peritoneal macrophages from CBA mice. Histograms presenting the distribution of rupture forces obtained for bacterial antigen – macrophage receptor systems taken from one of five independent experiments. (A) Histogram for EPS 115 covered tip and macrophages. (B) Histogram for EPS 142 covered tip and macrophages. (C) Histogram for EPS 148 covered tip and macrophages

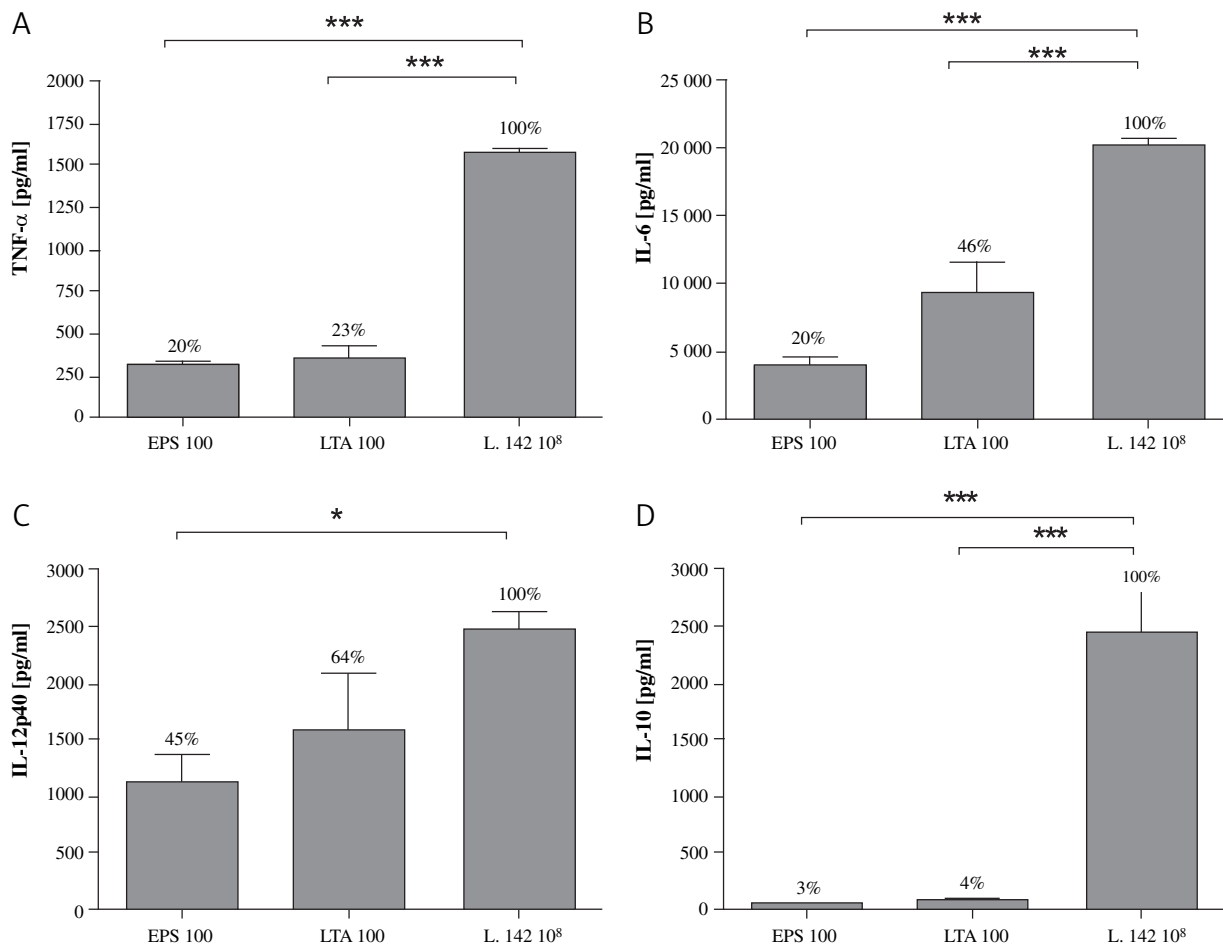


Fig. 5. Cytokine secretion from macrophages induced by LTA 142, EPS 142 and *L. johnsonii* 142. Peritoneal macrophages (5×10^5 /well) were incubated with LTA 142 (100 μ g/ml), EPS 142 (100 μ g/ml) or with bacterial concentrations at 10^8 cfu/well. The levels of TNF- α (A), IL-6 (B), IL-12p40 (C) and IL-10 (D) were measured by ELISA in the culture supernatants after 24 h of incubation. Data are mean \pm SEM values derived from at least three independent experiments, each based on macrophages isolated from four mice and tested in duplicate; * $p < 0.05$, *** $p < 0.001$

these EPSs to overall production of cytokines induced by lactobacilli seems to be negligible.

In conclusion, our studies confirm that immunostimulatory properties of lactobacilli are strain specific and this observation relates to whole bacteria and to bacterial components, such as LTA or EPS. These data are consistent with the increasing information about the role of probiotic bacteria (*Lactobacillus* spp.) to modulate innate immunity of the host. However, for the clinical application of *Lactobacillus* or its components, it is absolutely necessary to better understand the nature of interactions between bacterial biofilm and the immune cells *in vivo*.

Acknowledgments

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